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Note

Sensitive gas chromatographic method for the determination of diazepam and N-desmethyldiazepam in plasma

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Diazepam is a tranquillizer of the benzodiazepine type and is widely used for the symptomatic relief of anxiety, insomnia, psychiatric disturbances, seizures, and as preoperative medication [1].

Diazepam and its metabolites have been studied more intensively than the other benzodiazepines [2, 3].

Various papers have been published on the determination of diazepam and its metabolites by methods including spectrophotometry, gas chromatography with flame ionization or electron-capture detection, high-performance liquid chromatography, thin-layer chromatography, polarography and radioimmunoassay [2—4]. Gas—liquid chromatography (GLC) has been used extensively in the analysis of the benzodiazepines.

Chromatography at low concentrations, such as those found in blood and saliva following a single therapeutic dose, requires the use of an electron-capture detector to obtain good sensitivity. At the nanogram level, column adsorption processes, especially with the N-desalkyl compounds, become a problem. Such adsorbed compounds exhibit long retention times or do not elute from the column at all [5]. For these reasons, some GLC methods involve chromatography of the benzophenone hydrolysis products rather than of the benzodiazepines themselves [6—9]. These methods are time-consuming because of the clean-up procedure involved. Another disadvantage is their lack of specificity, due to the fact that metabolites of the parent drug if present in sufficient amounts would also yield the same benzophenone derivative [3]. By derivatizing the functional group of the intact compound, polarity is

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decreased and volatility is increased, thus providing increased sensitivity together with a shorter analysis time [10].

In this paper we describe a sensitive and specific procedure for the analysis of diazepam and N-desmethyldiazepam by electron-capture gas chromatography (EC-GC) after N-butylation of N-desmethyldiazepam based on a method reported by Greeley [11] for barbiturates. The method described below was developed to obtain a reliable analysis of diazepam and N-desmethyldiazepam at the nanogram level in dose—response studies. Our procedure is based on an EC-GC procedure which employs a liquid phase of 3% OV-17 and prazepam as internal standard, and requires a minimal amount of sample clean-up prior to EC-GC analysis. The method described provides adequate sensitivity for therapeutic monitoring of diazepam and N-desmethyldiazepam.

EXPERIMENTAL

Materials

All solvents were analytical-reagent grade and all inorganic reagents were prepared in distilled water. Buffer solution of pH 9 was prepared by mixing 37.5 ml of 0.1 *M* HCl with 0.05 *M* sodium borate to 250 ml final volume. Diazepam and N-desmethyldiazepam were kindly supplied by Hoffmann-La Roche Nederland, Mijdrecht, The Netherlands). Prazepam was supplied as a gift from Warner-Lambert Nederland, Mijdrecht, The Netherlands). Stock solutions of diazepam, N-desmethyldiazepam and prazepam were prepared by dissolving 10 mg of each compound in 100 ml of methanol; standard solutions were obtained by diluting these stock solutions to a concentration of 10 μg/ml. These standard solutions were diluted with plasma to concentrations covering the range 50—600 ng/ml in order to obtain calibration graphs.

Tetrabutylammonium hydroxide (TBAH) 0.2 *M* was prepared as follows. To a solution of 0.6 g tetrabutylammonium iodide in 10 ml of methanol, 0.5 g of silver oxide was added and shaken gently for 2 h at room temperature. After centrifugation the liquid phase was stored in a dark container at ca. 4°.

Samples

Blood samples drawn from patients (some on chronic administration of three times a day 2—5 mg diazepam) 2—3 h after an oral dose of 5 or 10 mg diazepam were taken with lithium heparin as anticoagulant and immediately stored at 4°. Separated plasma was frozen at —20° until analyzed.

Extraction

Plasma samples (0.5 ml) were spiked with 40.0 μl internal standard solution (400 ng), mixed with buffer (1.0 ml) and adjusted to pH 9 if necessary. This mixture was extracted by shaking it on a Vortex Genie mixer for 10 min with 5.0 ml of toluene—heptane (90:10). After centrifugation (2500 *g*) for 10 min, the organic phase (4.0 ml) was separated and transferred to a 10-ml conical-bottomed flask and evaporated under reduced pressure in a Büchi Rotavapor at 60°. Next, the residue was evaporated to complete dryness under a stream of nitrogen for 10 min at 50°. The residue was then dissolved in

50 μl of methanol and the butylation of N-desmethyldiazepam was performed as follows. To the 50 μl methanolic solution of the residue 4.0 μl of N,N-dimethylacetamide, 5.0 μl of TBAH (0.2 *M*) diluted (1:1) with methanol, and 100 μl of 1-iodobutane were added. Then the solution was mixed thoroughly on a Vortex Genie mixer for 10 sec. This mixture was allowed to react completely at room temperature for 10 min. After evaporation under a stream of nitrogen at 75°, the residue was dissolved in 500 μl of toluene. Volumes of 1 μl were injected into the gas chromatograph. The solution was stable for at least one week when stored in a refrigerator.

Gas chromatography

A Varian Model 3700 gas chromatograph equipped with an Aerograph® ^{63}Ni pulsed electron-capture detector and a Varian Model A 25 1-mV recorder was used. A Varian CDS 111 Chromatography Integrator was employed for measurement of peak retention times and peak areas. A coiled glass column (1.8 m × 3 mm I.D.) packed with 3% OV-17 on Chromosorb W HP (80–100 mesh) was used. The injection port was maintained at 290°, the column oven at 265°, and the detector at 300°. Pre-purified nitrogen was used as the carrier gas, at a flow-rate of 40 ml/min.

RESULTS

Recovery and precision

The recovery of internal standard from plasma (400 ng/ml) was 96 ± 7% (mean ± standard deviation; $n = 12$).

The over-all recoveries of diazepam (97 ± 8%) and N-desmethyldiazepam (as N-butyl derivative) (100 ± 12%) were calculated over the concentration range 50–600 ng/ml at 50, 250 and 600 ng/ml. The calibration graphs were constructed from three replicate measurements of five concentrations over that range (calibration graphs for diazepam, $y = 1.0869 \cdot 10^{-3}\ x - 2.2963 \cdot 10^{-2}$, $r = 0.996$; N-desmethyldiazepam as N-butyl derivative, $y = 1.7023 \cdot 10^{-3}\ x - 2.6617 \cdot 10^{-2}$, $r = 0.997$; concentration of internal standard; 400 ng/ml).

Within-run and between-run precision (four intervals of three days) were established. Diazepam and N-desmethyldiazepam were added to plasma at three different concentrations: 50, 250, and 600 ng/ml. The within-run precision of diazepam and N-desmethyldiazepam showed coefficients of variation varying from 5.4 to 1.6% and 10.5 to 5.9%, respectively, for this range. The between-run precision of diazepam and N-desmethyldiazepam showed coefficients of variation varying from 7.4 to 5.2% and 9.9 to 3.7%, respectively, for this range. The data on the precision are summarized in Table I.

Determination in plasma

Under the gas chromatographic conditions used, diazepam, N-desmethyldiazepam and the internal standard, prazepam, were eluted with retention times relative to prazepam of 0.63, 0.79, and 1.0, respectively (Fig. 1). A chromatogram obtained after analysis of a spiked plasma sample containing 400 ng/ml of each compound is given in Fig. 1b. Fig. 1a and c show chromatograms of blank and sample extracts of plasma. The limit of detection for

TABLE I

PRECISION DATA FOR DIAZEPAM AND N-DESMETHYLDIAZEPAM IN PLASMA

Drug	Within-run (n = 4)		Between-run (n = 4)	
	ng/ml (± S.D.)	C.V. (%)	ng/ml (± S.D.)	C.V. (%)
Diazepam	50 ± 3	5.4	51 ± 4	7.4
	234 ± 6	2.6	223 ± 14	6.3
	658 ± 11	1.6	637 ± 33	5.2
N-Desmethyldiazepam	49 ± 5	10.5	47 ± 5	9.9
	225 ± 7	3.2	220 ± 8	3.8
	611 ± 36	5.9	625 ± 23	3.7

diazepam and N-desmethyldiazepam (as N-butyl derivative) was 10 and 5 ng/ml, respectively, for 1-ml plasma samples.

DISCUSSION

The method reported here is based on the optimal extractability of diazepam and N-desmethyldiazepam into toluene—heptane at pH 9 [12]. The choice of an OV-17 liquid phase is based on its well-documented use in the quantitation of benzodiazepines utilizing gas chromatography. In a recent paper McAllister [13] showed the advantage of using prazepam as internal standard in the estimation of diazepam in plasma. It is assumed that high recoveries of both compounds, which are of comparable magnitude, indicate the reliability of the internal standard as such.

Rutherford [14] also used prazepam as the internal standard on the liquid phase OV-17. This column was deactivated every 4 h by means of 3-μl injections of a 5 g/l solution of dipalmitoyl phosphatidylcholine in ethanol. This treatment decreased the retention time of N-desmethyldiazepam by approximately 10% and the broad, tailing peak initially obtained became sharp and almost symmetrical. It is known that the tailing effect of standard solutions is diminished in the presence of a blood extract, probably due to the formation of an adsorption complex of the extracted blood lipids with active sites on the column [5]. A solution of cholesterol in acetone is also used as a column conditioner. In our experience these methods for deactivating adsorption sites on the column did not provide us with the required reproducibility for the chromatography of N-desmethyldiazepam.

The analytical method described above has an advantage over previous reported methods because of its great sensitivity for N-desmethyldiazepam.

Derivatization by means of alkylation at position N_1—H was employed in order to reduce adsorption and yield symmetrical peaks. The results obtained by conversion to the trimethylsilyl derivative at the N_1 position, as reported by Greaves [15], did not provide us with reliable results. Flash-heater alkylation of N-desmethyldiazepam to obtain a methyl, ethyl, propyl or butyl derivative, based on methods reported by Kowblansky et al. [16] and Pecci and Giovanniello [17], for the determination of xanthines and barbiturates, was not reproducible. During these experiments we found that the N-butyl

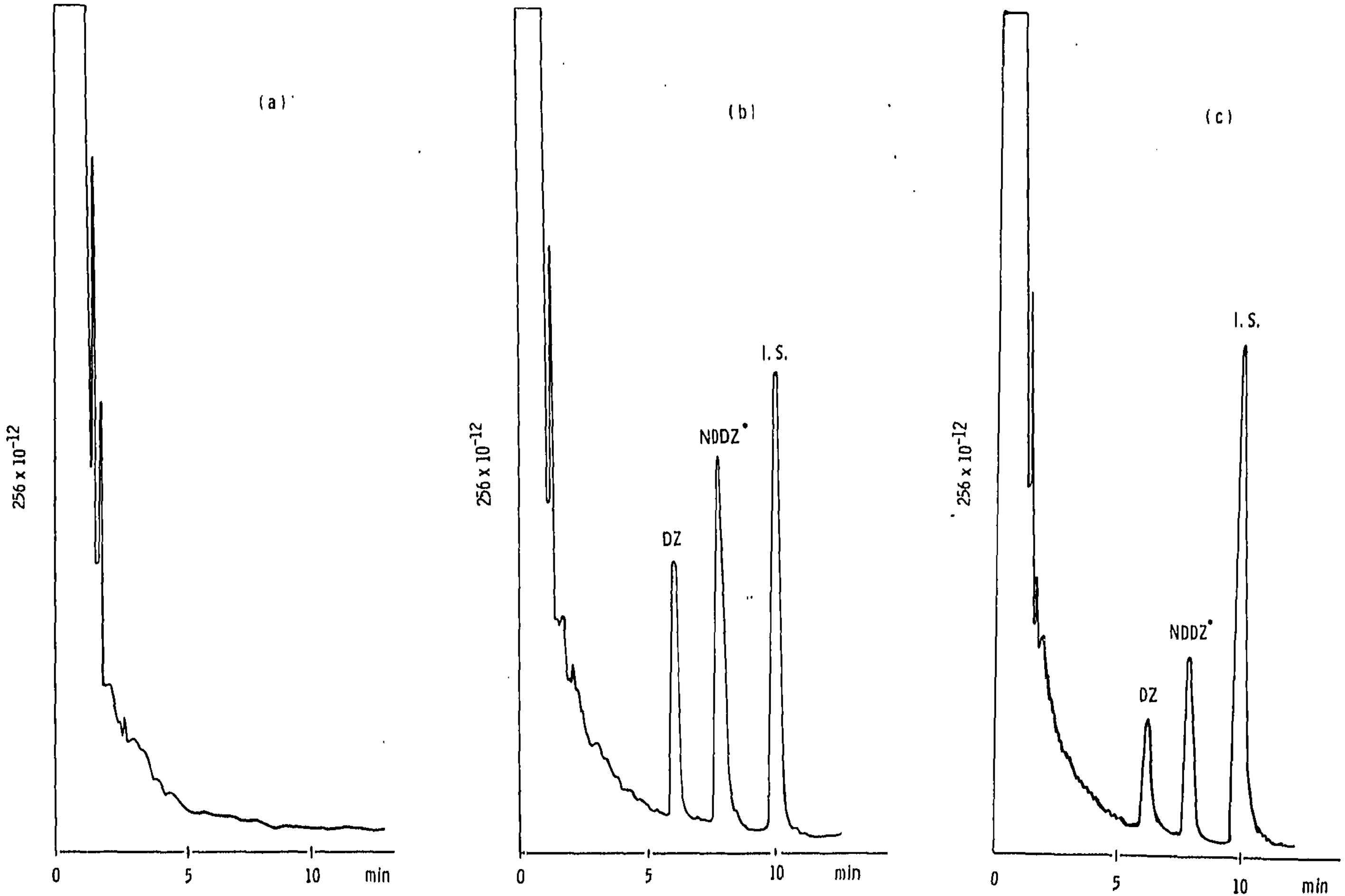

Fig. 1. Gas chromatograms of (a) plasma blank; (b) spiked plasma sample containing diazepam (DZ) 400 ng/ml, N-desmethyldiazepam (as N-butyl derivative, NDDZ*), and prazepam (as internal standard, I.S.); (c) plasma sample obtained from a patient (on chronic administration of three times a day 2 mg diazepam) 2.5 h after an oral dose of 5 mg diazepam, containing DZ 178 ng/ml and NDDZ* 192 ng/ml.

derivative yielded good separation. Finally, the procedure reported by Greeley [11] for alkylation of barbiturates has proved extremely successful for the preparation of a volatile N-butyl derivative of N-desmethyldiazepam for gas chromatographic analysis.

This method, with minor modifications, has also been used successfully to determine concentrations of diazepam and N-desmethyldiazepam in saliva [18].

Compared with the high-performance liquid chromatographic method recently reported by Brodie et al. [19] the procedure described here has comparable sensitivity and selectivity.

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